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Novel fibro-inflammation markers in assessing left atrial remodeling in non-valvular atrial fibrillation

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Background: Structural remodeling is associated with the fibroinflammatory process in the atrial extracellular matrix. In the present study we aimed to investigate whether serum levels of new circulating remodeling markers differ in patients with atrial fibrillation (AF) compared to patients with sinus rhythm.





Material/Methods: The study population included 52 patients diagnosed with non-valvular AF and 33 age-matched patients with sinus rhythm. Serum levels of Galectin-3, matrix metalloproteinase-9 (MMP-9), lipocalin-2 (Lcn2/NGAL), N-terminal propeptide of type III procollagen (PIIINP), Hs-Crp, and neutrophil-to-lymphocyte ratio (NLR) were measured. The left atrial volume (LAV) was calculated by echocardiographic method and LAV index was calculated.

Results: Galectin-3, MMP-9, and PIIINP levels were significantly higher in AF patients except NGAL levels (1166 pg/ml (1126–1204) and 1204 pg/ml (1166–1362) $p=0.001$, 104 (81–179) pg/ml and 404 (162–564) pg/ml $p<0.0001$, and 1101 (500–1960) pg/ml and 6710 (2370–9950) pg/ml $p<0.0001$, respectively). The NLR and Hs-CRP levels were also higher in AF (2.1 ± 1.0 and 2.7 ± 1.1 $p=0.02$ and 4.2 ± 1.9 mg/L and 6.0 ± 4.7 mg/L $p=0.04$, respectively). In correlation analyses, NLR showed a strongly significant correlation with LAVi, but Hs-CRP did not ($p=0.007$ $r=0.247$, Pearson test and $p=0.808$ $r=0.025$, Pearson test, respectively). Moreover, Galectin-3, MMP-9, and PIIINP had a strong positive correlation with LAVi ($p=0.021$ $r=0.640$, Spearman test and $p=0.004$ $r=0.319$ Pearson test, and $p=0.004$ $r=0.325$ Pearson test, respectively).

Conclusions: Novel fibrosis and inflammation markers in AF are correlated with atrial remodeling. Several unexplained mechanisms of atrial remodeling remain, but the present study has taken the first step in elucidating the mechanisms involving fibrosis and inflammation markers.

MeSH Keywords: **Atrial Fibrillation • Atrial Remodeling • Collagen Type III • Galectin 3 • Lipocalins • Matrix Metalloproteinase 9**

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Background

Atrial fibrillation (AF) is the most common cardiac arrhythmia and increases the risk of stroke and death [1]. Inflammation is an important factor related to the initiation, maintenance, and recurrence of AF. This abnormal inflammation may cause a prothrombotic state, finally resulting in thromboembolism [2]. In persistent AF, atrial enlargement and structural and electrical remodeling form the basis of atrial changes. Structural remodeling is associated with an activated and gradually progressive fibrosis and inflammation process in the atrial extracellular matrix (ECM) [3–5]. Matrix metalloproteinases (MMPs) are major mediators of normal ECM remodeling [3–5, 7]. Moreover, studies have increased our understanding of the role of inflammation in AF by showing that C-reactive protein (CRP) [6–8] and the neutrophil-to-lymphocyte ratio (NLR) are associated with AF [9–11].

However, the exact mechanism of the pathogenesis and progression of AF remains to be elucidated. Various mediators may play a role in the pathogenesis. A growing body of evidence is showing that galectin-3 [12–14], lipocalin-2/neutrophil gelatinase-B-associated lipocalin (Lcn2/NGAL) [15–17] and N-terminal propeptide of type III procollagen (PIIINP) [18–20] seem to play important roles in the cardiovascular inflammation and fibrosis that result in cardiac remodeling.

In the present study, we aimed to investigate whether serum levels of galectin-3, Lcn2/NGAL, PIIINP, and NLR differ in patients with AF compared with patients with a sinus rhythm, with guidance of known markers such as serum MMP-9 and Hs-CRP levels. We also evaluated the associations of these markers with atrial structural remodeling, which was interpreted by measuring the left atrial volume index.

Material and Methods

Patient selection

The study population included 85 patients who were seen in our outpatient clinic between March 2012 and January 2013. Fifty-two patients diagnosed with non-valvular (mitral and aortic valve) persistent AF (AF duration longer than 1 month) were recruited into the AF group. End-stage hepatic or renal disease, malignancy, any prior blood transfusions, carotid artery disease, prior transient ischemic attack and ischemic or hemorrhagic stroke, oral anticoagulant usage, and NYHA functional class III or IV patient were exclusion criteria in our study. Thirty-three age-matched patients with sinus rhythm were recruited into the control group. A clinical history of risk factors such as diabetes mellitus, hypertension, hypercholesterolemia, and cardiovascular disease were recorded. For each patient, we calculated height, weight, body mass index (BMI), and body surface area (BSA). Based on the

serum creatinine level on admission, estimated glomerular filtration rate (e-GFR) was calculated by using the Modification of Diet in Renal Disease (MDRD) formula. The ethical implications regarding the study were approved by the local ethics committee and informed consent was obtained from each patient.

Echocardiographic assessment

The LV ejection fraction (EF) was assessed by the modified biplane Simpson method. Cardiac dimensions were measured according to the recommendations of the American Society of Echocardiography by M-mode and 2-dimensional echo [21]. LV mass (LVM) was calculated from 2D echocardiographic measurements by using Devereux formula: $LVM = 1.04 * [(LVsw + LVpw + LVEDD)^3 - (LVEDD)^3] - 13.6$ and was indexed to body surface area [22]. Two cardiologists, blinded to patient clinical history, performed the physical examinations, measured outcome variables, interpreted all echocardiograms, and verified LV

volumetric analyses. Left ventricular ejection fraction (LVEF) was calculated using the biplane method of discs (modified Simpson's rule) in the apical 4- and 2-chamber views at end systole and end diastole. Measurements were obtained as the mean value from the apical 4- and 2-chamber views. The left atrial volume was calculated by using the biplane method of discs (modified Simpson's rule) in the apical 4- and 2-chamber views at end diastole of the atria. Measurements were obtained as the mean value from the apical 4- and 2-chamber views. The LAVI was then calculated as LAV divided by body surface area [4,23].

Blood samples and biochemical measurements

Venous blood samples were drawn after an outpatient clinical examination. The samples were kept at room temperature for 15 min to permit coagulation and were then centrifuged at $1000 \times g$ for 15 min. The serum was separated with the aid of a pipette, transferred to Eppendorf tubes, and kept at $-40^{\circ}C$ until analysis.

The serum levels of galectin-3, MMP-9, Lcn2/NGAL, and PIIINP were measured using commercial enzyme-linked immunoassay kits, and each assay was carried out in duplicate. The galectin-3 level was determined using sandwich ELISA (Human Galectin-3 ELISA kit; eBioscience), NGAL levels (Human Lipocalin-2/NGAL ELISA kit; BioVendor Research and Diagnostic Products), MMP-9 levels (Human Matrix Metalloproteinase 9; Bio-Medical Assay), and a PIIINP kit (Human Procollagen III N-Terminal Propeptide; Bio-Medical Assay). The minimal measurable concentrations for these detection systems are 120 pg/ml for galectin-3, 20 pg/ml for NGAL, 60 pg/ml for PIIINP, and 50 pg/ml for MMP-9.

The hemoglobin level and white blood cell count were determined as part of the automated complete blood count using a

Sysmex XT-1800i (USA) hematology analyzer. The baseline NLR level was measured by dividing the neutrophil count by the lymphocyte count. A white blood cell count of $>12,000$ cells/ μ l or $<4,000$ cells/ μ l and high body temperature of $>38^{\circ}\text{C}$ were excluded from the study to ensure a subclinical inflammatory status.

Statistical analyses

Continuous variables are expressed as mean \pm SD or median (interquartile range) when appropriate. Categorical variables are expressed as percentages. To compare parametric continuous variables, Student's *t*-test was used; to compare nonparametric continuous variables, the Mann-Whitney U-test was used. To compare categorical variables, the chi-square-test was used. The Pearson and Spearman correlation coefficient were used to determine parametric and nonparametric measure of statistical dependence between 2 variables. Multivariate regression analysis was used to identify the independent predictors of higher LAVi value >48 mm³/m² (mean LAVi value is 48 mm³/m²). All variables showing significance values of less than 0.1 on univariate analysis were included in the model. A 2-tailed P-value of less than 0.05 was considered to indicate statistical significance. The statistical analyses were performed using software (SPSS 15.0, SPSS Inc, Chicago, IL).

Results

Baseline characteristics

The baseline characteristics of the groups (mean age, 71 \pm 8 years; minimum age, 42 years; maximum age, 85 years; 62% female) are presented in Table 1. There were no differences between the groups in terms of baseline characteristics, excluding congestive heart failure, and no differences in the conventional laboratory findings. Aspirin and digitalis use was significantly higher in the NVAf group, but there were no differences in the remaining medications.

Echocardiographic measurements

The EF was significantly lower and the LVMass, LAV, and LAVi were significantly higher in patients with NVAf (Table 1).

Inflammatory and remodeling markers

There were significant differences between the groups in terms of inflammatory and remodeling markers, with the exception of the NGAL levels (Table 2). The galectin-3, MMP-9, and PIIINP levels were significantly higher in patients with NVAf (1166 pg/ml (1126–1204) and 1204 pg/ml (1166–1362) $p=0.001$, 104 (81–179) pg/ml and 404 (162–564) pg/ml $p<0.0001$, and 1101

(500–1960) pg/ml and 6710 (2370–9950) pg/ml $p<0.0001$ respectively, Mann-Whitney U test for all. The NLR and Hs-CRP levels were also higher in patients with NVAf (2.1 \pm 1.0 vs. 2.7 \pm 1.1 mg/l, $p=0.02$, Student's *t*-test and 4.2 \pm 1.9 vs. 6.0 \pm 4.7 mg/l, $p=0.04$, Student's *t*-test, respectively) (Figures 1 and 2).

Figures 3 and 4 demonstrate a correlation between LAVi and the inflammatory and remodeling markers as shown by the correlation analyses. NLR showed a significant correlation with LAVi, whereas Hs-CRP did not ($p=0.007$, $r=0.247$, Pearson's test and $p=0.808$, $r=0.025$, Pearson's test, respectively). Moreover, galectin-3, MMP-9, and PIIINP showed a strong positive correlation with LAVi ($p=0.021$, $r=0.640$, Spearman's test; $p=0.004$, $r=0.319$, Pearson's test; and $p=0.004$, $r=0.325$, Pearson's test, respectively).

In univariate analysis, a cut point was determined as mean LAVi value, MMP-9, PIIINP, NLR, and EF were correlated with high LAVi (LAVi >48 mm³/m²) (Table-3). In multivariate analysis, PIIINP [odds ratio (OR)=1.22, 95% confidence interval (CI) (1.11–1.41), $P=0.001$] was the only independent factor associated with high LAVi.

Discussion

The most important finding of our study is the new fibrosis and inflammation markers in patients with AF: galectin-3, PNIINP, Lcn2/NGAL, Hs-CRP, and NLR. We have shown higher levels of these fibrosis and inflammation markers with the guidance of the serum levels of MMP-9, Hs-CRP, and echocardiographic measurements such as LAVi in patients with AF compared with patients with a sinus rhythm.

ECM components and turnover are regulated by MMPs [4]. During collagen synthesis and degradation, MMPs are upregulated, indicating that the unbalanced expression of the MMP/TIMP system may have a role in the process of atrial structural remodeling. Structural remodeling can be shown using the echocardiographic measurement LAV/LAVi [4,23]. We found higher levels of MMP-9 in patients with AF. Our findings confirm those of previous studies in which enhanced MMP-9 activity was proposed as a major molecular mechanism contributing to the dilation of the atria [4,24,25].

The role of galectin-3 in the pathogenesis of cardiac fibrosis involves the recruitment of additional macrophages, myofibroblasts, and fibroblasts, resulting in cellular proliferation and secretion of procollagen after mechanical and neurohormonal stimuli, which results in secretion of galectin-3 from macrophages [26]. Galectin-3 has been shown to mediate cell-to-cell and cell-to-ECM interactions and acts as a novel chemo-attractant for monocytes and macrophages [12,26]. To the best of

Table 1. Baseline characteristics of groups.

Variables	Control group (n: 33)	AF group (n: 52)	P-value
Patients characteristics			
Age	70±10	70±10	0.942
Female/Male n	20/13	34/18	0.656
BMI kg/m ²	29.5±4.4	30±5.2	0.620
BSA m ²	1.87±0.21	1.86±0.22	0.934
Diabetes n	9/33	11/52	0.603
Hypertension n	17/33	39/52	0.035
Hyperlipidemia n	7/33	5/52	0.135
CHF n	3/33	12/52	0.005
CAD n	7/33	12/52	0.842
Laboratory			
Creatinine mg/dl	1.0±0.3	1.0±0.3	0.808
eGFR mL/min	72±20	69±20	0.491
LDL mg/dl	120±30	116±38	0.703
Hb gr/dl	13.1±1.8	13.3±1.6	0.625
Platelet ×10 ³	247±67	231±60	0.265
Echocardiography			
EF%	63±5	52±13	<0.001
LVMass gr	221±49	256±65	0.02
LAV mean mm ³	57±21	100±40	<0.0001
LAVi mm ³ /m ²	31±10	54±22	<0.0001
Admission medication			
Aspirin	12/33	31/52	0.037
Beta blocker	7/33	26/52	0.008
Digitals	0/33	9/52	0.011
Ace/ARB	14/33	27/52	0.393
Statin	7/33	5/52	0.135
Diuretics	3/33	10/52	0.206
Clopidogrel	1/33	1/52	0.743
CaCB	4/33	12/52	0.206
OAD	7/33	8/52	0.492
Insulin	1/33	1/52	0.743
NSAI	5/33	14/52	0.062

ACE – Angiotensin converting enzyme; ARB – angiotensin receptor blocker; BMI – body mass index; BSA – body surface area; CHF – congestive heart failure; CAD – coronary artery disease; NLR – neutrophil to lymphocyte ratio; NSE – neuron specific enolase; Hs-CRP – high sensitive C reactive protein; MPV – mean platelet volume; eGFR – estimated glomerular filtration rate; EF – ejection fraction; LV – left ventricle; LAV – left atrial volume; LAVi – left atrial volume index; CaCB – Ca channel blocker; OAD – oral anti-diabetics; NSAI – non-steroid anti-inflammatory. Results are expressed as mean ±SD or frequency (with in group percentage) and median (Interquartile range). Student-t and Chi-square-test are used.

Table 2. Values of fibro-inflammatory markers of groups.

Variables	Control group (n: 33)	AF group (n: 52)	P-value*
Hs-CRP mg/L	4.2±1.9	6.0 ±4.7	0.04
NLR	2.1±1.0	2.7±1.1	0.02
Galectin-3 pg/ml	1166 (1126–1204)	1204 (1166–1362)	0.001#
Lipocalin-2/NGAL pg/ml	132±63	152±55	0.138
MMP-9 pg/ml	104 (81–179)	404 (162–564)	<0.0001#
PIIINP pg/ml	1101 (500–1960)	6710 (2370–9950)	<0.0001#

NLR – neutrophil to lymphocyte ratio; NGAL – neutrophil gelatinase-associated lipocalin; Hs-CRP – high sensitive C reactive protein; MMP-9 – matrix metalloproteinase-9; PIIINP – procollagen III N-terminal propeptide. Results are expressed as mean ±SD and median (interquartile range). * Student-t test; # Mann-Whitney U test.

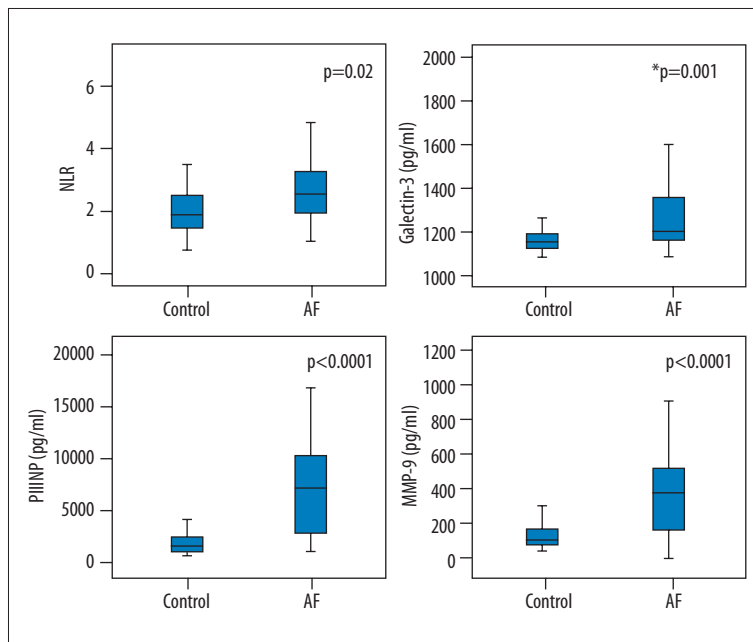


Figure 1. Mean values of fibrosis and inflammation markers in groups: MMP-9 – matrix metalloproteinase-9, PIIINP – N-terminal propeptide of type III procollagen, NLR – neutrophil-to-lymphocyte ratio (Student-t test was used), and Galectin-3 (Mann-Whitney U test was used).

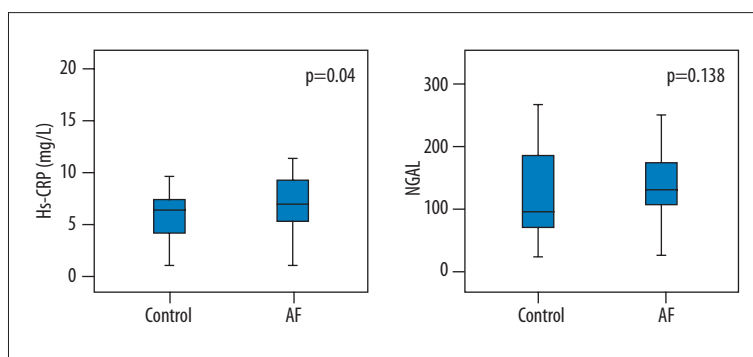


Figure 2. Mean values of fibrosis and inflammation markers in groups: Hs-CRP – high sensitive CRP, Lcn2/NGAL – lipocalin-2/neutrophil gelatinase-B associated lipocalin (Student-t test was used for both).

our knowledge, galectin-3 levels have not been evaluated in patients with AF, especially with LAVi. We found higher levels of galectin-3 in patients with AF than in the control group, indicating that galectin-3 may play a crucial role in the migration of inflammatory cells. Based on these data, we suggest that

galectin-3 can be used as a novel target in patients with AF to decrease the degree of fibrosis and inflammation in the atria.

Lipocalin-2/NGAL is a proinflammatory adipokine [27]. Cardiomyocytes, vascular wall cells, and fibroblasts in myocarditis

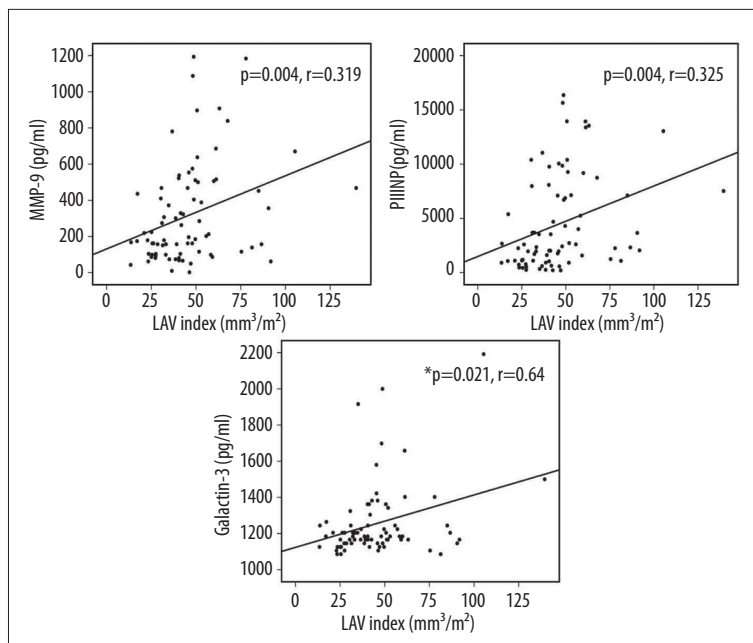


Figure 3. Correlation analyses between LAVi (Left Atrial Volume Index) and fibrosis and inflammation markers: MMP-9 – Matrix metalloproteinase-9, PIIINP – N-terminal propeptide of type III procollagen, Pearson correlation analysis was used for both, Galectin-3 (Spearman correlation analysis was used). Figure reflects the entire (patient and control) group.

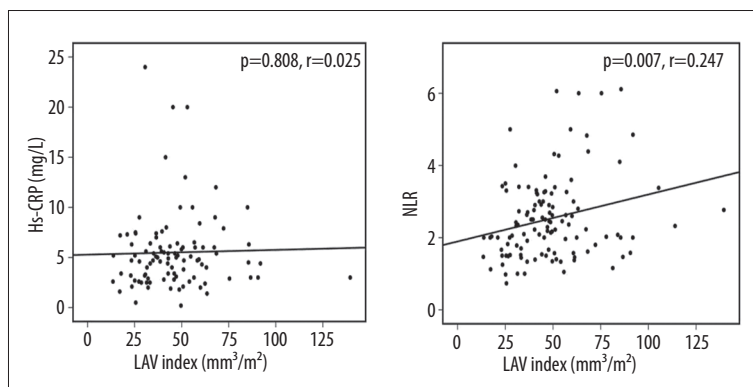


Figure 4. Correlation analyses between LAVi (Left Atrial Volume Index) and fibrosis and inflammation: Hs-CRP – high sensitive CRP, NLR – neutrophil to lymphocyte ratio, Pearson correlation analysis was used for both. Figure reflects the entire (patient and control) group.

strongly express lipocalin-2/NGAL, which is associated with insulin resistance [16]. Lipocalin-2/NGAL has been studied in various cardiovascular diseases (e.g., myocarditis and coronary heart disease) but not yet in AF [17]. We found no significant difference in the levels of lipocalin-2/NGAL between patients with AF and the control group. There are several possible explanations for this. Lipocalin-2/NGAL levels are generally higher in obese patients [15], and in our patient population there was no significant difference in the mean body mass index. In addition, statin use reduces the plasma lipocalin-2/NGAL level [28]. Therefore, in this study, statin use in the control group was slightly higher.

One of the serum markers of collagen synthesis is PIIINP, which reflects collagen turnover. Its role as a marker of collagen synthesis has been shown in various cardiovascular diseases, but to date few study in AF has been performed [18–20,29,30]. Swartz et al. have demonstrated that PIIINP levels correlate with presence of LA fibrosis and may be a predictor for post-operative AF [30]. PIIINP is a cardiac remodeling marker and is used to monitor

the anti-remodeling effect of various drugs such as aldosterone antagonists [31,32]. In this study, we showed significantly higher levels of PIIINP in patients with AF compared with the control group, indicating that during collagen turnover, PIIINP may play a role in the remodeling of the atria in patients with AF.

Previous studies have contributed to our understanding of the role of inflammation in AF by showing that Hs-CRP [6–8,33] and NLR are associated with AF [9–11]. According to the review by Bhat et al., the association of NLR with AF has not been evaluated in detail. Recently, in association between NLR and AF, few studies have focussed on cardioversion success, ablation therapy results, and thromboembolic stroke risk [34]. In our study, NLR showed a significant correlation with LAVi, whereas Hs-CRP did not. According to our study results, NLR is an inflammatory marker that plays a role in remodeling of the atria. On a molecular and cellular level, galectin-3 and various other markers activate and increase the numbers of monocytes and macrophages involved in cell-to-cell and cell-to-ECM interactions during atrial remodeling.

Table 3. Independent predictors of LAVi (>48 mm³m²) in multivariate regression analysis.

Variables	Univariate OR (95% CI)	P-value	Multivariate OR (95% CI)	P-value
Age	–	0.309		
Female	1.19 (0.47–2.99)	0.453		
Diabetes mellitus	0.78 (0.34–1.8)	0.577		
Hypertension	0.74 (0.33–1.6)	0.466		
Coronary Artery Disease	0.97 (0.33–2.80)	0.831		
E-GFR	–	0.146		
EF	–	0.001		
Hs-CRP	–	0.724		
NLR	–	0.034		
Lipocalin-2/NGAL	–	0.589		
Galectin-3	–	0.132		
MMP-9	–	0.001		
PIIINP	–	0.001	1.22 (1.11–1.41)	0.001

EF – ejection fraction; LAVi – left atrial volume index; eGFR – estimated glomerular filtration rate; NLR – neutrophil to lymphocyte ratio; NGAL – neutrophil gelatinase-associated lipocalin; Hs-CRP – high sensitive C reactive protein; MMP-9 – matrix metalloproteinase-9; PIIINP – procollagen III N-terminal propeptide; CI – confidence interval; OR – odds ratio.

The renin-angiotensin-aldosterone system plays an important role in structural atrial remodeling [35]. The current literature indicates that angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) prevent the development of AF (primary prevention) [36,37]. Contrary to these data, the significance of the role of ACEIs and ARBs in upstream therapy (secondary prevention) is not clear. Some results have been encouraging, whereas others have been very unfavorable; however, some data show a positive anti-remodeling effect in patients with AF [38,39]. In light of recent reports, and according to our data, PIIINP, galectin-3, and NLR levels could be important in determining the presence of underlying heart disease (remodeling status) in patients who are candidates for medical therapy. A reduction in new-onset AF has been reported in patients with remarkable underlying heart disease treated with ACEIs or ARBs, but the benefit was minimal in patients with moderate structural heart disease and recurrent AF. A GISSI-AF echocardiographic sub-study revealed that during 1 year ARB for did not reverse LA remodeling or prevent AF recurrence [23]. Therefore, early anti-remodeling therapy should be recommend to patients with persistent AF before irreversible ECM remodeling occurs, as pointed-out by Gramley et al. [3]. It is important to identify the time at which atrial remodeling starts in patients likely to develop AF in the short term. Camm et al. reported a purpose for new clinical concepts of AF in the facilitation of intensive clinical monitoring and management [40]. Based on our results, we suggest that LAVi and new fibrosis and inflammation markers such as galectin-3, PIIINP, and NLR may predict the beginning of the atrial remodeling process.

Study limitations

Two limitations must be considered. The main limitation is the lack of histologic correlation, because we did not perform a histopathological examination of atrial tissue. Also, our study included a small population. However, research on fibrosis and inflammation markers at the tissue level would contribute additional information.

Conclusions

Certain fibrosis and inflammation markers in AF are correlated with atrial remodeling. Several unexplained mechanisms of atrial remodeling remain, but the present study has taken the first step in elucidating the mechanisms involving fibrosis and inflammation markers. Although targeting fibrosis and inflammation as early anti-remodeling therapy is not supported by the current data, present study findings also suggest that galectin-3, PIIINP, and NLR may contribute to the clinical and therapeutic management of AF as novel targets for therapies that aim to decrease fibrosis and inflammation in the atria.

Declaration of conflicting interest

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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